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A reassessment of the origin and distribution of the subterranean genus *Pseudolimnocythere* Klie, 1938 (Ostracoda, Loxoconchidae), with description of two new species from Italy

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Abstract

Groundwater ecosystems host a rich and unique, but still largely unexplored and undescribed, biodiversity. Several lineages of ostracod crustaceans have subterranean representatives or are exclusively living in groundwaters. The stygobitic genus *Pseudolimnocythere* Klie, 1938 has a West Palearctic distribution, and includes few living and fossil species of marine origin. Through a comprehensive literature review and the description of the two new living species, *Pseudolimnocythere abdita* **sp. nov.** and *Pseudolimnocythere sofiae* **sp. nov.**, from springs in the Northern Apennines, Italy, a morphological analysis was carried out with the aim of comparing the valve morphology of living and fossil species, and to discuss previous hypotheses about time and mode of colonization of inland waters. *Pseudolimnocythere* species show a low variability in valve morphology, with a remarkable stasis over geological times. The distribution of extant and fossil species is consistent with a scenario of multiple and independent events of colonization of continental habitats linked to sea level variations starting from Middle Miocene in the Paratethys and, later, in the Mediterranean. The most common colonization routes of inland waters have taken place through karst formations along ancient coastlines, although we cannot exclude some minor active migration through the hyporheic zone of streams. Available distribution data suggest a poor dispersal ability of *Pseudolimnocythere* species after they had colonized continental waters.

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Keywords
colonization, evolution, stygobitic, morphology, taxonomy

Introduction

Although groundwater ecosystems form the largest freshwater biome (Griebler et al. 2014), they belong to the least explored habitats on earth (Ficetola et al. 2019). Many taxonomic lineages have a high biodiversity and are often represented by several endemic or rare species in groundwater (Bregović et al. 2019; Bishop et al. 2020), but their actual species richness, distribution patterns, and the ecological determinants shaping their areals are still poorly known (Deharveng et al. 2009; Stoch and Galassi 2010; Eme et al. 2015; Zagmajster et al. 2018). Ninety-five percent of global freshwater (excluding the polar ice caps) is stored in the continental subsurface constituting a major source of ecosystem services (Griebler et al. 2014). However, at the same time, subterranean ecosystems are threatened by different anthropogenic impacts and by a general inadequacy of protection policies, conditions that seriously endanger their biological diversity and the ecosystem services they provide (Boulton et al. 2008; Mammola et al. 2019). Improving our knowledge of groundwater fauna and its origin is thus of paramount importance for implementing effective conservation practices.

Crustacea is by far the most diversified stygobitic taxon in Europe, contributing to about 70% of the overall groundwater species richness (Zagmajster et al. 2018). The study of crustacean fauna has been especially important in developing models of colonization of continental subterranean waters (Stoch and Galassi 2010; Bauzà-Ribot et al. 2012; Delić et al. 2020). In particular, the class Ostracoda has been used to investigate time and mode of evolutionary radiation and colonization patterns (Danielopol 1980; Danielopol et al. 1994; Horne 2003).

It is well documented that stygobionts (i.e., taxa occurring exclusively in groundwater during their entire life cycle) can have a double origin (Coineau and Boutin 1992): limnicoid stygobionts derive from epigean freshwater ancestors, while thalassoid stygobionts derive directly from marine ancestors. In the last case, pre-adapted marine species (Iglikowska and Pawłowska 2015), living in the interstitial or in fissured rocky habitats, could have crossed the salinity boundary via an intermediate mixo- to oligohaline coastal zone, and eventually have migrated in inland groundwaters (Boutin and Coineau 1990; Noteboom 1991). It has been suggested that between 9 and 12 independent invasions of freshwaters by marine ostracods have occurred, the first possibly in the early Carboniferous (Iglikowska 2014; Iglikowska and Pawłowska 2015).

The class Ostracoda consists of bivalved crustaceans with a laterally compressed body. Their calcitic carapaces are the most abundantly preserved arthropod remains in the fossil record (Matzke-Karasz and Smith 2020). Ostracods occur in almost all aquatic and in some semi-terrestrial habitats (Smith et al. 2015). Following Meisch et al. (2019), freshwaters host 2,330 accepted species of ostracods assigned to 270 genera of the order Pocodocopida, living both in surface than subterranean waters. In recent years, several papers reported the

description of the poorly known subterranean ostracod faunas from different parts of the world (e.g., Karanovic 2007; Reeves et al. 2007; Smith 2011; Peterson et al. 2013; Iepure et al. 2016; Külköylüoğlu et al. 2017; Mazzini et al. 2017; Pociecha et al. 2021).

The ostracod family Loxoconchidae has living representatives in marine, brackish and freshwater habitats (Athersuch and Horne 1984). Recent non-marine Loxoconchidae have a Palearctic distribution; ten species in five genera are currently known, accounting for c. 0.4% of the total freshwater ostracod diversity (Meisch et al. 2019). Within Loxoconchidae, the stygobitic genus *Pseudolimnocythere* Klie, 1938 has a West Palearctic distribution, and includes few Recent and fossil freshwater species of marine origin. Danielopol (1980) argued that the genus *Pseudolimnocythere* was closely related to the marine interstitial genus *Tuberoloxoconcha* Hartmann 1974 (the most notable difference between the species of the two genera is the length of the antennular distal article), the latter genus placed in the subfamily Pseudolimnocytherinae Hartmann and Puri 1974 within the Loxoconchidae. Danielopol (1979) accomodated the genus *Pseudolimnocythere* within the tribe Pseudolimnocytherini. The subfamily Pseudolimnocytherinae and the tribe Pseudolimnocytherini are no longer recognized as valid after the results of a cladistic analysis, based on morphocharacters of Recent freshwater ostracods, which showed that *Pseudolimnocythere* and *Loxoconcha* Sars, 1866 form a more derived clade within the family Loxoconchidae (Savatenalinton and Martens 2009). Besides its taxonomic interest, the genus *Pseudolimnocythere* has been the subject of several studies aimed at investigating the evolution of subterranean ostracods and their colonization modes (Danielopol 1977, 1979, 1980; Danielopol and Bonaduce 1990).

In this paper, after a comprehensive literature review and the description of two new living species from springs in the Northern Apennines, Italy, we critically revise the previously proposed scenarios in the light of the new available data and offer a morphological analysis of all living and fossil *Pseudolimnocythere* species known so far. After the taxonomic analysis, we used morphological and biogeographic tools to elucidate the origin and colonization of continental waters by this genus. We tested two different hypotheses: (i) a "single marine invasion" where pseudolimnocytherids obtained their current distribution through dispersal, mainly in interstitial habitats; (ii) a "multiple marine invasion" where a previous dispersal along marine coasts is followed by a very limited dispersal in freshwaters and a long history of speciation events due to vicariance.

Materials and methods

Ostracod specimens described in this paper were sampled from the Cirone rheocrene spring and the Poiano spring system (Emilia Romagna region) (Suppl. material 1: Fig. S1). In the Cirone spring, a baited trap was used, as described in Bottazzi et al. (2011). In the Poiano spring system, a drift net was positioned to filter the entire discharge of the major spring mouth.

Ostracods were preserved in ethanol 75%. Dissections were done under a stereomicroscope (Zeiss 47 50 22). Valves were examined by Scanning Electron Microscopy (SEM) using a Philips XL-30 and a FEI Quanta 400, and then stored dry in micropaleontological

slides; soft parts were dissected in glycerine, mounted in glycerine on a microscope glass slide and sealed using nail polish. Drawings of soft parts were made with the aid of a camera lucida attached to a compound microscope (Zeiss 47 30 11-9901).

Chaetotaxy of the limbs in descriptions follows Meisch (2000), and higher taxonomy of the Ostracoda is according to Meisch et al. (2019).

The outlines of valves obtained both from SEM images and drawings taken from literature, were gathered using GIMP (GNU Image Manipulation Program, https://www.gimp.org), then saved as TPS file using the TPSutil32 v. 1.76 software and digitized using the TPSdig v. 2.20 software (Rohlf 2009). Superimposition of valve outlines was performed using Morphomatica 1.6.0.1 (Linhart et al. 2007), a program designed for the approximation of ostracod specimens using an adapted B-splines algorithm. For the approximation method in Morphomatica, 16 arbitrary control points were considered with 6 iteration steps.

Results

Taxonomic account

Class Ostracoda Latreille, 1802
Subclass Podocopa Sars, 1866
Suborder Cypridocopina Baird, 1845
Order Podocopida Sars, 1866
Superfamily Cytheroidea Baird, 1850
Family Loxoconchidae Sars, 1925

Genus *Pseudolimnocythere* Klie, 1938

Diagnosis (modified from Danielopol 1979 and Karanovic 2012). Small ostracods (length < 0.4 mm), without sexual dimorphism. Carapace ornamented with rounded pits. Hinge henodont (single posterior tooth in RV), invicidont, or amphidont. Calcified inner lamella wide, marginal pore canals branched; normal pore canals with small sieve plates located in deep funnels. Antennula, six-segmented (fourth and fifth segments only partially separated or completely indistinct), the distal segment exceeding the length of the second segment. Exopod of antenna not segmented; penultimate segment with two basal setae and one aesthetasc, and two distal (anterior) setae.

***Pseudolimnocythere abdita* sp. nov.**
http://zoobank.org/6EA43D77-BD52-461F-B5CF-266AD34C9E79
Figs 1, 2, 3A, B

Type locality. Rheocrene spring Cirone, road from Bosco di Corniglio to Cirone pass, municipality of Corniglio, province of Parma, Emilia-Romagna region, coordinates

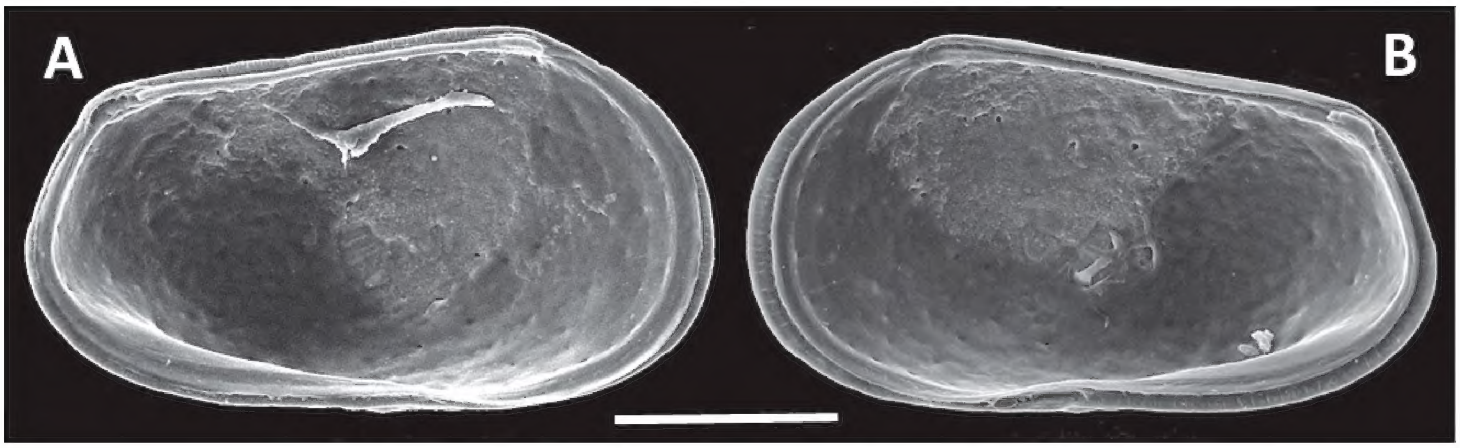

Figure 1. *Pseudolimnocythere abdita* sp. nov., VP1152, adult ♀ **A** left valve, internal view **B** right valve, internal view. Scale bar: 100 μm.

44°26'59"N, 10°00'38"E, 1126 m a.s.l. (Suppl. material 1: Figs S1, S4A). Natural free-flowing spring located on shales and limestone (Suppl. material 1: Fig. S2), estimated average discharge 2 l s^{-1}, water temperature 7.9–8.5 °C, electric conductivity c. 360 μS cm^{-1}, pH 7.5–7.7 (Bottazzi et al. 2011). Sample collected by Elisa Bottazzi on May 15, 2007.

Material investigated. One adult female designated as holotype, with valves stored dry in a micropaleontological slide (used for SEM) and soft parts dissected in glycerine and kept in a sealed slide (VP1152). Previously reported as *Pseudolimnocythere* cf. *hypogea* Klie, 1938 in Bottazzi et al. (2008, 2011) and *P.* sp. 1 in Pieri et al. (2015). Material deposited in the Ostracod Collection of the Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma.

Derivation of name. From the Latin adjective abditus-a-um, meaning hidden, concealed, but also mysterious, due to the fact that only one individual of this species was collected despite repeated samplings in the type locality and in other springs of the same region.

Description. Valves (Figs 1, 3A, B). Subrectangular in lateral view. Anterior margin broadly rounded, posterior margin rounded and blunty pointed. Surface ornate with fossae. Hinge reduced invicidont: LV with smooth bar, smooth anterior tooth and tripartite posterior socket; RV with smooth groove and anterior socket, posterior tooth consisting of a large element and two smaller teeth towards the anterior part. Muscle scars not visible.

Appendages (Fig. 2). Antennula six-segmented. Second podomere with setulae on anterior margin and a ventro-apical seta slightly longer than the next segment; third podomere short, bearing a dorso-apical seta about two times its length; fourth segment formed by fourth and fifth podomeres partially fused, with one posterior and one anterior subequal setae inserted near fusion line, and distally one posterior seta and three anterior setae reaching or slightly beyond tip of next segment; terminal podomere long and thin, distally with a seta fused at the base with aesthetasc y_a, and a free seta of the same length as the latter. Antenna with robust basipodite. Spinneret seta (exopodite) gently arched proximally, central part nearly straight, distal end decidedly bent and with pointed tip almost reaching distal end of terminal

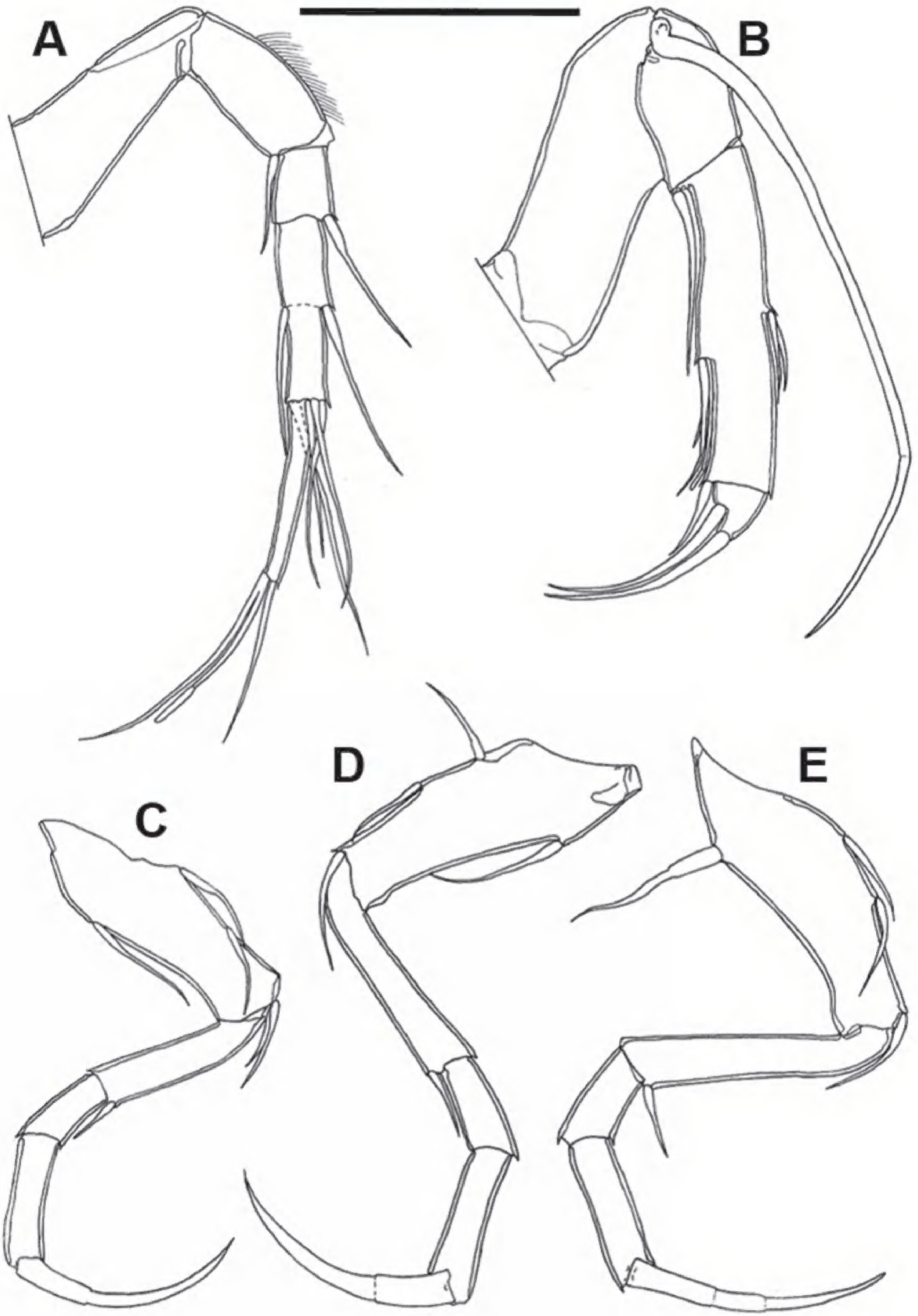

Figure 2. *Pseudolimnocythere abdita* sp. nov., VP1152, adult ♀ **A** antennula **B** antenna **C** first thoracopod **D** second thoracopod **E** third thoracopod. Scale bar: 50 μm.

claws. Endopodite four-segmented. First podomere with a ventro-apical setae reaching slightly beyond mid-length of next segment. Penultimate segment consisting of second and third segments fully fused, with two short setae inserted halfway the length of anterior margin, and two unequal setae and aesthetasc Y at c. 4/7 of posterior margin, tip of the latter slightly surpassing segment distal end, and one robust ventro-apical seta; last segment with two claws, one longer inserted more proximally and the other apically. Thoracopods (walking legs) four-segmented, first podomere sturdier and more enlarged than following ones. First segment of first thoracopod with four setae in anterior position, one proximally, one medially and two distally, and a posterior seta inserted proximally; second podomere with a dorso-apical seta shorter than next segment; third and fourth segment without setae; last podomere distally bearing a robust claw with proximal third enlarged. Second and third thoracopod with analogous setal arrangement; first segment with three anterior setae and a longer posterior seta; second segment with a dorso-apical seta, third segment without setae; terminal claw of second thoracopod more enlarged and stouter than in other thoracopods, that of third thoracopod consisting of three parts with gradually decreasing diameter. Male unknown.

Measurements. Valve length 308 μm, height 173 μm (n = 1).

Distribution. The species is known from its type locality only.

Differential diagnosis. *Pseudolimnocythere abdita* sp. nov. differs from other described living or subfossil species of the genus with "sloping" valves (see below) in its intermediate size, being larger than *P.* sp. sensu Schornikov, 2013 (c. 0.25 mm) and *P.* sp. Montanari et al. 2021 (c. 0.28 mm), and shorter than *P.* sp. Peterson et al. 2013 (c. 0.35 mm), and *P.* sp. Danielopol 1980 (0.34–0.36 mm). For all these species, only the morphology of the valves is known, therefore a comparison with the soft parts of *P. abdita* sp. nov. is not possible.

Remarks. *Pseudolimnocythere abdita* sp. nov. is here formally described, in spite of the fact that a single female specimen was available. We have decided to do this for the following reasons:

- both valve and soft features are described, allowing us to clearly distinguish the new species from its congenerics;
- so far only two other living species are known for the genus *Pseudolimnocythere*, which is of particular importance to better understand the origin, phylogenetic affinities and distribution of non-marine representatives of the family Loxoconchidae;
- the habitat from which the species was collected strikingly differs from those of other living congeners (see below);
- further samplings performed in the type locality and surrounding spring areas did not yield additional specimens.

We are thus confident that *Pseudolimnocythere abdita* sp. nov. can be unambiguously identified on the characters presented here.

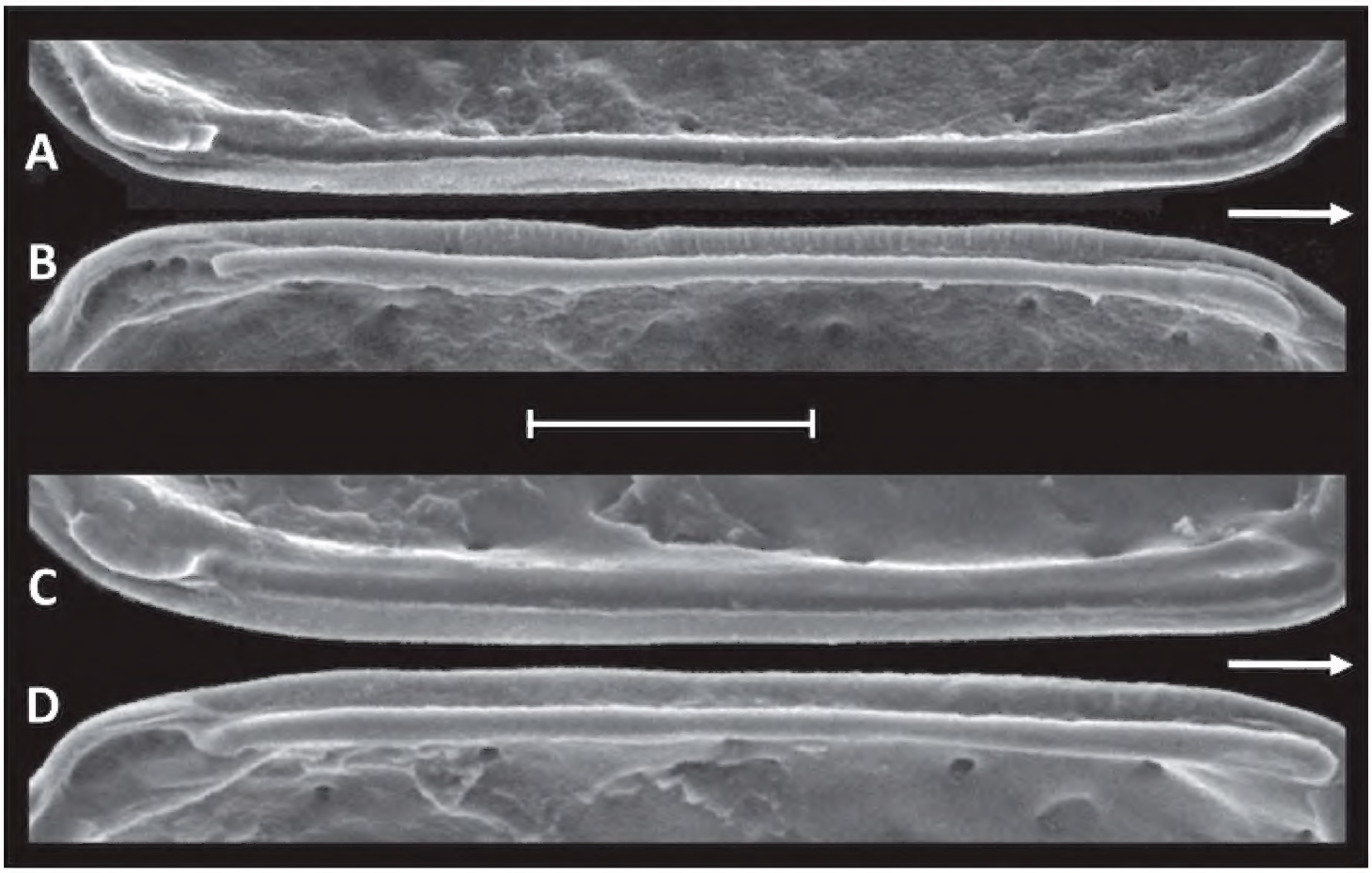

Figure 3. A, B *Pseudolimnocythere abdita* sp. nov., VP1152, adult ♀ **C, D** *Pseudolimnocythere sofiae* sp. nov., VP1125, adult ♂ **A** right valve, internal view, detail dorsal margin **B** left valve, internal view, detail dorsal margin **D** right valve, internal view, detail dorsal margin **E** left valve, internal view, detail dorsal margin. Scale bar: 40 μm.

***Pseudolimnocythere sofiae* sp. nov.**
http://zoobank.org/060FAE40-34C2-45BE-885F-D26F96B8ADF8
Figs 3C, D, 4, 5

Type locality. Poiano springs, Upper Secchia Valley, municipality of Villa Minozzo, province of Reggio nell'Emilia, Emilia-Romagna region, coordinates 44°23'31"N, 10°26'20"E, 423 m a.s.l. (Suppl. material 1: Figs S1, S4B). Average discharge > 400 l s^{-1}, water temperature between 8.9 and 10.8 °C, conductivity ranging from ~9 to ~17 mS cm^{-1} (Stoch et al. 2009a). Poiano is the major spring complex of a large outcrop of Upper Triassic evaporites, comprising a sequence of gypsum-anhydrite and dolostone beds with local salt bodies (Suppl. material 1: Fig. S3). The spring drains an aquifer of unique properties composed of anhydrite with halite lenses at depth and gypsum at the surface (both with high NaCl content) (Chiesi et al. 2010). Sample collected by Fabio Stoch on December 21, 2015.

Material investigated. More than 30 specimens from the type locality and sample of the holotype, and additional specimens from nearby localities (see below). Previously reported as *Pseudolimnocythere* sp. in Stoch et al. (2009a, b) and *P.* sp. 1 in Pieri et al. (2015).

Holotype. GR804, adult ♂, soft parts dissected in glycerine in a sealed slide, valves used for SEM imaging and stored dry in a micropalaentological slide.

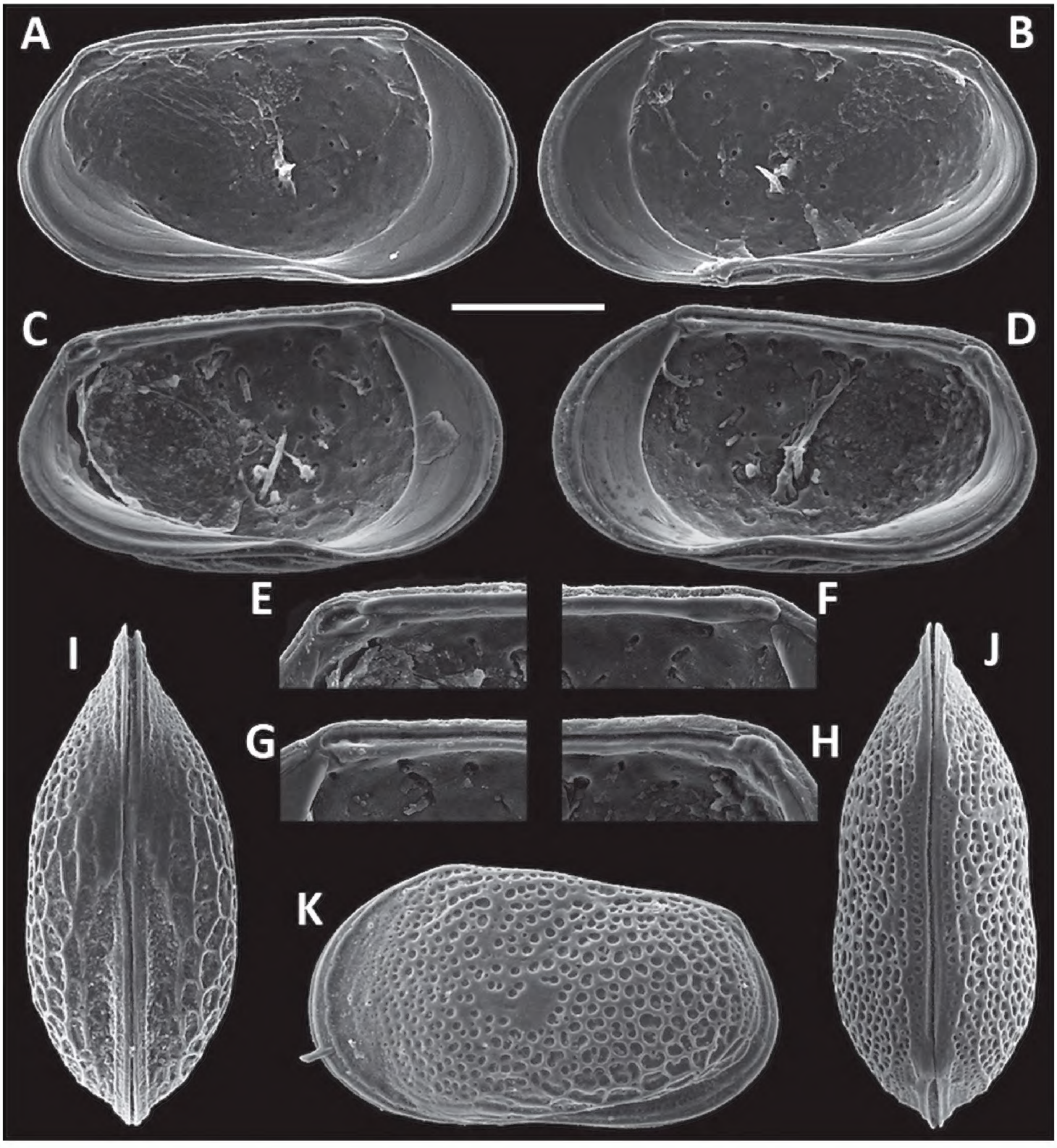

Figure 4. *Pseudolimnocythere sofiae* sp. nov. **A, B** VP1125, adult ♂ **C–H** GR797, adult ♀ **I** VP1120, adult (sex undetermined) **J** VP1122, adult (sex undetermined) **K** VP1121, adult (sex undetermined) **A** left valve, internal view **B** right valve, internal view **C** left valve, internal view **D** right valve, internal view **E** left valve, internal view, detail postero-ventral corner **F** left valve, internal view, detail antero-dorsal corner **G** right valve, internal view, detail antero-dorsal corner **H** right valve, internal view, detail postero-ventral corner **I** carapace, dorsal view **J** carapace, ventral view **K** carapace, left lateral view. Scale bar: 100 μm (**A–D, I–K**); 66 μm (**E–H**).

Derivation of name. This species is dedicated to GR's daughter, Sofia Rossetti Tekleab. Furthermore, among the different meanings of the ancient Greek noun "σοφία" there are also "knowledge" and "wisdom". Our hope is that the description of this new species will shed more light on morphological characteristics and evolutionary relationships of the genus *Pseudolimnocythere*.

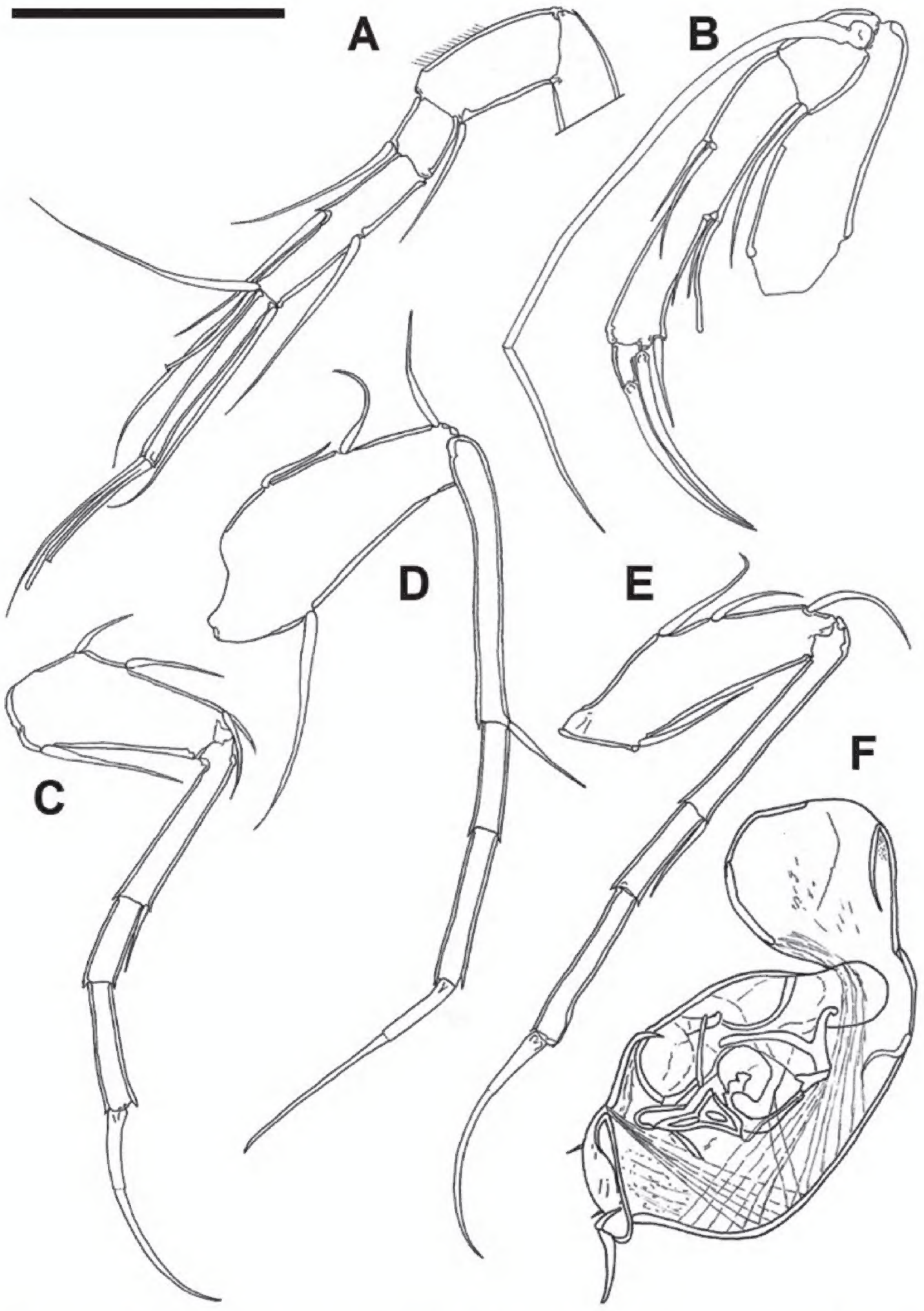

Figure 5. *Pseudolimnocythere sofiae* sp. nov., GR804, adult ♂ **A** antennula **B** antenna **C** first thoracopod **D** second thoracopod **E** third thoracopod **D** hemipenis. Scale bar: 50 μm (**A, E**); 64 μm (**F**).

Description. Valves (Figs 3C, D, 4). Carapace small, in dorsal view elliptical, valves sub-rectangular in lateral view. Viewed laterally the dorsal edge is sloping gently posteriorly turning into a curve. Anterior end of the carapace broad and rounded. Maximum length at mid height, maximum height in the anterior third. Surface ornamented with subrounded pits, with dimensions increasing towards posterior. In dorsal view, anterior end beak shaped. In internal view: vestibulum well developed, selvage strong and narrow, line of concrescence well developed. Dorsal margin straight, ventral margin concave in the middle part, with an additional closing mechanism where the RV overlaps the LV. Hinge amphidont: LV with smooth cardinal bar swelling anteriorly into a bi or trilobate tooth, posteriorly with a lower small tooth and an upper large socket with several lobes, RV with complementary intercardinal groove, swelling anteriorly into two-three sockets, posterior element with a multilobate tooth. Muscle scars typical of the family: four adductor muscle scars in a vertical row. Sexual dimorphism not very pronounced, with males slightly larger and stouter than females. Average carapace length 0.32 mm.

Appendages (Fig. 5). Antennula slender, six-segmented. Second podomere with short setulae on the second half of the anterior margin and a ventro-apical seta reaching c. 1/3 of the length of next segment; third podomere short, with a dorso-apical seta slightly shorter than next segment; penultimate segment consisting of fourth and fifth podomeres fused, with two subequal setae, one posterior and one anterior, at junction of the two fused segments, and distally one posterior seta and three anterior setae, the longer ones reaching beyond tip of next segment; terminal podomere long and thin, distally with a free seta and aesthetasc y_a fused at the base with a seta. Antenna with stout, trapezoidal basipodite. Spinneret seta (exopodite) reaching tip of distal claws of endopodite, proximally bowed and nearly straight in the middle part, distal end folded and thin. Endopodite four-segmented; first segment of endopodite with a ventro-apical setae slightly exceeding mid-length of next segment; penultimate segment formed by second and third podomeres fused, with two setae of different length at c. 1/3 of anterior margin, aesthetasc Y and two shorter setae at about half length of posterior margin, and one ventro-apical seta; last segment with two claws, the longest one proximo-posterior and the other distal. Thoracopods (walking legs) four-segmented, with first podomere stout and remaining ones long and slender with straight margins. First segment of first thoracopod with four anterior setae, one proximal, two median and one distal, and a proximo-posterior seta reaching the end of the segment; second podomere with a dorso-apical seta; third and fourth segment without setae; last segment with a distal claw weakly subvided in two parts, the distal one c. 2/3 of the length and narrower. Same setal formula in second and third thoracopod: first segment with three anterior setae and a longer posterior seta; second segment with dorso-apical seta; third segment with no setae; last segment with long claws. Hemipenis with chitinized anterior lobe having a narrow base and distally wider and sub-quadrate.

Measurements. Valve length 303–331 μm (n = 9).

Distribution. In addition to individuals drifted from the Poiano spring, rare specimens of *Pseudolimnocythere sofiae* sp. nov. were found in some nearby habitats reported by Stoch et al. (2009b): in the interstitial of Secchia river (where spring waters come

out), Sologno stream and Lucola stream; in the gypsum caves named Risorgente di Ca' della Ghiaia (cadastral number 244 ER) and Tanone Grande della Gaggiolina (cadastral number 154 ER); no specimens were found in all other types of groundwater and interstitial habitats examined in the same area, or in springs located in soils on marly-arenaceous deposits upstream of the evaporite outcrops (Stoch et al. 2007, 2009b).

Differential diagnosis. *Pseudolimnocythere sofiae* sp. nov. is easily distinguishable from described congeneric species. The better described species is *P. hartmanni* Danielopol (1979) from which it differs in overall outline, size, ornamentation and development of the ventral posterior margin. *Pseudolimnocythere* sp. (Peterson et al., 2013, fig. 8U) seems much larger, although measurements were taken from the figure (where a LV is erroneously reported as a RV). *Pseudolimnocythere hypogaea* (sensu Karanovic and Pesce 2001, figs 1–6) is slightly smaller, stouter in the overall appearance and with a characteristic wavy dorsal margin. *Pseudolimnocythere* sp. (Danielopol 1980, fig. 11A–F) from the Skulijca cave displays a faint ornamentation and a stout anterior marginal rim; the overall external shape is not visible in the photos provided but in internal view the central curve of the ventral margin is less pronounced.

Pseudolimnocythere sp. (Peterson et al. 2013) is stouter, with a different surface ornamentation and an oblique dorsal margin. *Pseudolimnocythere* sp. sensu (Schornikov et al., 2014) is slightly smaller (302–311 μm), with a surface ornamentation occurring only in the marginal areas. The specimen illustrated in Schornikov et al. (fig. 4k, 2014) is probably a juvenile. The drawings of *P. hypogaea* by Klie (1938) and Karanovic and Pesce (2001) clearly display a sinuous dorsal margin and the lack of the additional closing mechanism in the ventral area. The morphology of the hemipenis differs markedly from that of the extant species of Pseudolimnocythere for which males have been described (Fig. 6).

Note. The distal segment of walking legs in *Pseudolimnocythere abdita* sp. nov. and *P. sofiae* sp. nov. is fused with the basal part of the claws (Figs 2C–E, 5C–E). The same pattern is reported for the Limnocytheridae as well (see Meisch 2000).

Other records of living and sub-Recent *Pseudolimnocythere*

Pseudolimnocythere sp. (fig. 8U in Peterson et al. 2013)

Frasassi cave complex (Marche, Italy), inner lakes of the Grotta del Fiume (cadastre number 8 Ma), in the remains of the subfossil eels at Lago delle Anguille; outside the cave, sulfidic springs on the bank of Sentino River (Fig. 9C). More than one thousand subfossil carapaces and valves recovered from various sites in the cave, possibly dating back 7,200 years (Mariani et al. 2007). Previously reported as *Pseudolimnocythere* cf. *P. hypogaea* by Peterson et al. (2009).

Pseudolimnocythere hypogaea Klie 1938

Slightly brackish groundwater habitats in Apulia, Italy, were cited as 'type localities' by Klie (1938): wells near Bari (Fig. 9E) and in two caves, L'Abisso (cadastre number 141

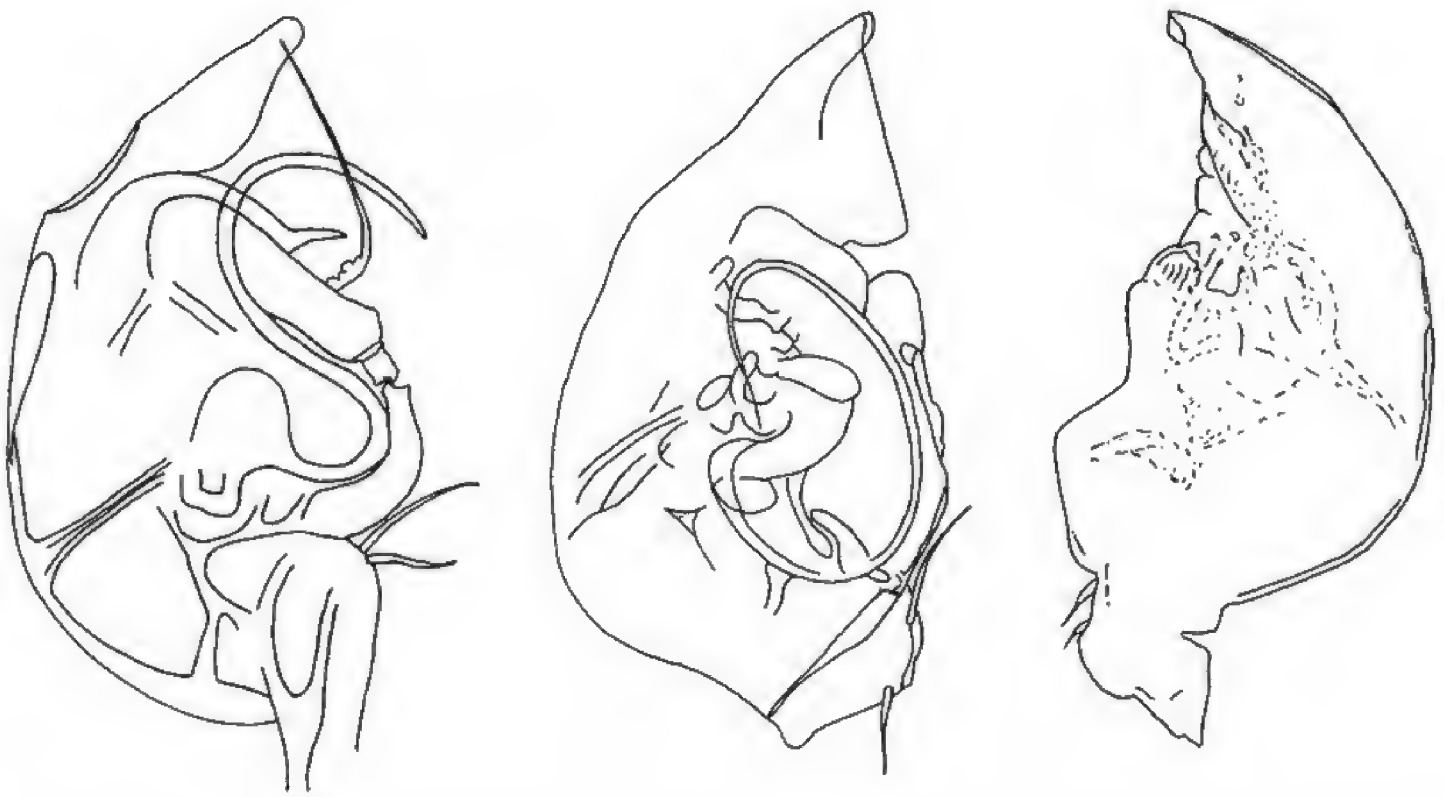

Figure 6. Hemipenis of Recent species of *Pseudolimnocythere* **A** *P. hypogaea* (redrawn from Klie, 1938) **B** *P. hypogaea* (redrawn from Karanovic and Pesce 2001) **C** *P. hartmanni* (redrawn from Danielopol 1979). Not to scale.

Pu, Castro Marina, Lecce) and Cunicolo dei Diavoli (101 Pu, Porto Badisco, Lecce), these latter located c. 10 km apart (Fig. 9F). Later found again (reported as *P. hypogea*) in L'Abisso cave, and in a well on the road Foggia-Manfredonia, Apulia (site PU56) (Fig. 9G) by Karanovic and Pesce (2001), who also gave a redescription of this species. L'Abisso cave is located a few dozen metres from the sea and its water is brackish, the salinity varying in relation to the seasonal mixing of seawater and groundwater (Inguscio et al. 2009). PU56 is defined as a "freshwater well" located on the Gargano promontory at about 10 km from the coast, but actually this area is characterised by a karst aquifer with brackish waters (FS, pers. obs.). Finally, the cave named Cunicolo dei Diavoli opens at the seashore, it is flooded by brackish as well as marine waters (being influenced by the effect of the tides). Karanovic and Pesce (2001, pages 22 and 23) remarked some differences in valve size and chaetotaxy with respects to the description of *P. hypogaea* by Klie (1938). Since in both cases the descriptions were made on material collected from different localities, the existence of different species, albeit strongly related to each other, cannot be excluded. Differences in valve size and outline between specimens illustrated by Klie (1938) and Karanovic and Pesce (2001) can be observed in Fig. 7C, D.

***Pseudolimnocythere* sp. (Danielopol and Bonaduce 1990, not figured)**

Interstitial of a temporary stream, beach in front of La Baillaury (site Ba-2), Bay of Banyuls, France (Fig. 9A). The salinity of the interstitial waters varies according to the stream flow (14‰ in May 1984). A single adult male, not figured.

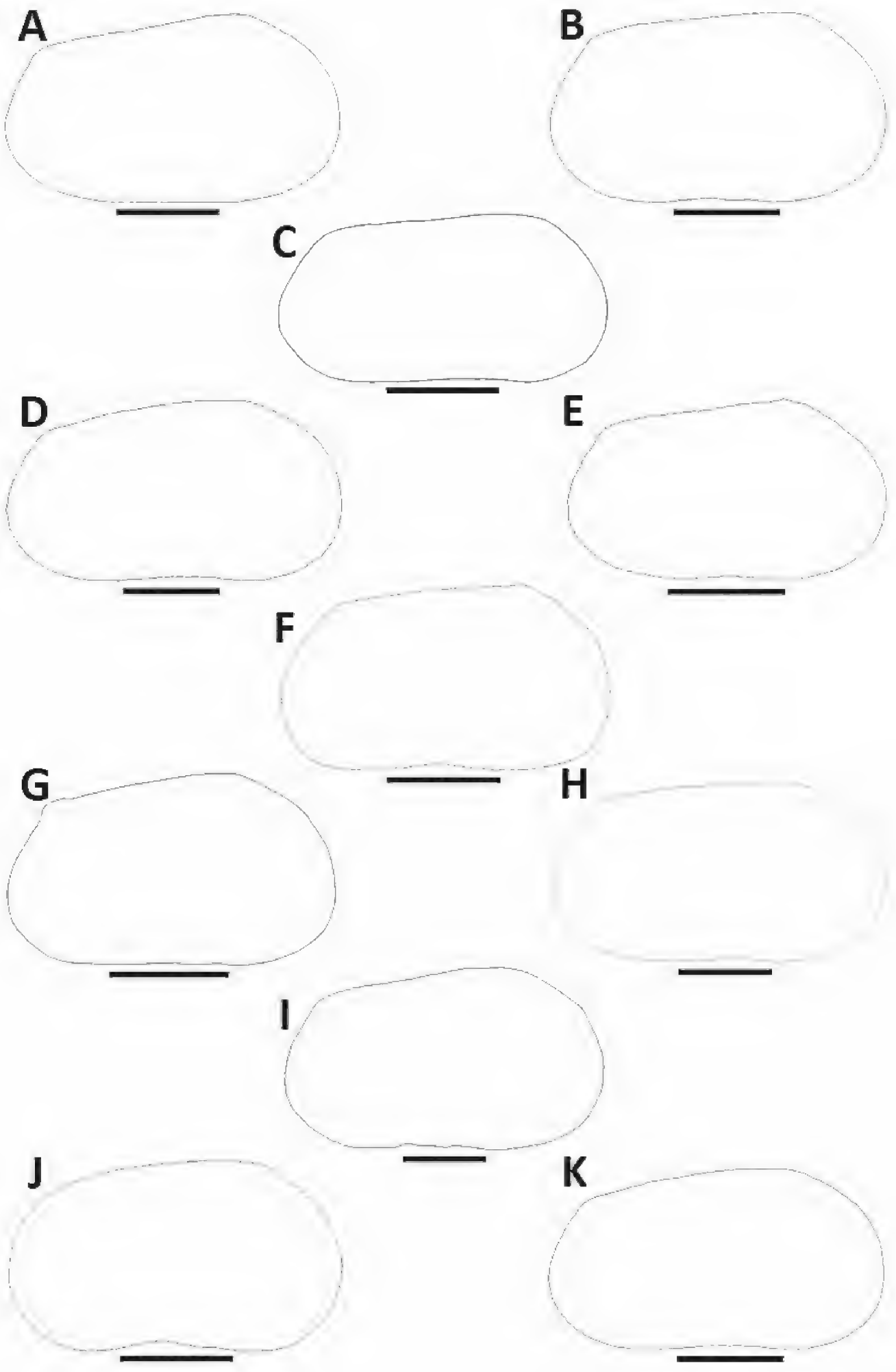

Figure 7. Valve outlines of Recent and fossil species of *Pseudolimnocythere* **A** *P. Pseudolimnocythere abdita* sp. nov. **B** *P. sofiae* sp. nov. **C** *P. hypogaea* (from Klie 1938) **D** *P. hypogaea* (from Karanovic and Pesce 2001) **E** *P.* sp. (from Peterson et al. 2013) **F** *P. hartmanni* (from Danielopol 1979) **G** *P.* sp. (from Danielopol 1980) **H** *P.* sp. (from Montanari et al. 2021) **I** *P.* sp. sensu Schornikov 2013 (from Schornikov et al. 2014) **J** *P. hainburgensis* (from Danielopol et al. 1991) **K** *P. hainburgensis* (from Gross and Piller 2006). Scale bar: 100 μm (in case of intraspecific variability in valve size, the maximum value is considered).

***Pseudolimnocythere* sp. (fig. 7G in Danielopol 1980)**

Cave Skuljica, Krk Island, Croatia (Fig. 9H). Only carapaces and valves, no living specimens.

Pseudolimnocythere hartmanni (fig. 3 in Danielopol 1979 and fig. 10A–H in Danielopol 1980)

A well fed by freshwater, 19 m from the seashore on the northwestern coast of Euboea (Evvia) Island, village Aghios Georghios, Greece (Fig. 9J).

***Pseudolimnocythere* sp. sensu Schornikov 2013 (Ivanova et al. 2014; figs 4I–N in Schornikov et al. 2014)**

Inner shelf of the northeastern coast of Black Sea, approximately 2.4 km from the town of Gelendzhik, and living in the freshwater underflow of the small Jeane River, 16 km upstream off the town of Gelendzhik (Fig. 7K). This species was first recorded as *Elofsonia*? sp. (Schornikov 2012).

?*Pseudolimnocythere* sp. A (figs 4o, p in Schornikov et al. 2014)

Black Sea, Tsemes Bay (town of Novorossiysk) at a depth of 2 m. The attribution of the figured specimens to the genus *Pseudolimnocythere* is doubtful and therefore no further considered herein.

***Pseudolimnocythere* sp. (Montanari et al. 2017, 2021)**

Vodeni Rat anchialine cave, 13 m above sea level, and about 30 m inland from the rocky coast, Sveti Klement Island, Pakleni Archipelago, southeastern of the island of Hvar (Croatia) (Fig. 9I). Karstic well with freshwater of meteoric origin until the 4th-7th century CE; today flooded by anchihaline waters, with a stratification with marine waters at the bottom (Montanari et al. 2021). Twenty-five well-preserved single valves, no soft parts nor paired and closed articulated valves.

Remarks

The described species of the genus *Pseudolimnocythere* show low variability in valve morphology, and for extant species the identification is mainly based on male copulatory organ morphology. This situation makes it difficult to identify and compare both fossil and Recent species exclusively through the morphological analysis of valves, or when male sexual characters cannot be examined in living species. Therefore, definite criteria for species assignment which are valid both in neontological and paleontological research are presently unavailable for this genus. Nevertheless, analysis of valve outlines in normalized area mode allowed to identify three distinct morphological groups. The first group, characterized by "curved" valves (rounded postero-dorsal margin, dorsal margin gently arched, convex ventral margin),

contains *P. hainburgensis* only; the second group, consisting of species with "sloping" valves (dorsal margin straight and decidedly sloping backward, in some species also a straight postero-dorsal margin), includes *P. abdita* sp. nov., *P.* sp. Peterson et al., 2013, *P.* sp. sensu Schornikov, 2013, *P.* sp. Danielopol, 1980 and *P.* sp. Montanari et al., 2021; the third group joins species with "slightly inclined" valves (dorsal margin straight, but less inclined backwards than in the previous group), namely *P. sofiae* sp. nov., *P. hypogaea* and *P. hartmanni* (Fig. 8). The three groups are here used for convenience, and no taxonomic value should be attributed to them. We did not consider it useful to proceed with a statistical analysis of the valve outlines. In fact, despite the supposed absence of a significant sexual dimorphism in the valve shape and the symmetry between right and left valve in *Pseudolimnocythere*, the scarcity of figured specimens - and sometimes also their low quality - in the literature forced us to compare outlines obtained by individuals of different gender, and in some cases after reversal of the valves. It is worth emphasizing that the three Recent species whose morphology of the male copulatory organs is known (*P. hypogaea*, *P. hartmanni* and *P. sofiae* sp. nov.) and which certainly constitute distinct taxonomic entities (Fig. 6), are found in the same cluster defined by valve shape. However, this does not happen for the specimens of *P. hypogaea* described by Karanovic and Pesce (2001) for which, as mentioned above, there is some uncertainty about their identification with the material examined and figured by Klie (1938). Another potentially applicable criterion is that based on difference in adult valve size: at the lower extreme of the range are *P.* sp. sensu Schornikov, 2013 (c. 0.25 mm) and *P.* sp. Montanari et al., 2021 (c. 0.28 mm), while *P.* sp. Danielopol (1980) (0.34–0.36 mm) and *P.* sp. Peterson et al. (2013) (c. 0.35 μm) define upper size limits; the remaining species have intermediate sizes (Fig. 7).

There are several differences in the morphology of the hinge among *Pseudolimnocythere* species. On the other hand, different types of these structures are known for the family Limnocytheridae, even at the genus level (Yamaguchi 2003, Savatenalinton and Martens 2009).

Records of fossil *Pseudolimnocythere*

***Pseudolimnocythere hainburgensis* (fig. 7I, J in Danielopol et al. 1991).**

Miocene (Badenian) of the Vienna Basin, Hainburg, Lower Austria (Fig. 9L). Gross and Piller (2006) further recorded *P. hainburgenis* from the same formation.

According to Danielopol et al. (1991), *P. hainburgenis* probably lived in fresh- or brackish water habitats since it was associated with Characeae gyrogonites and the freshwater ostracods *Darwinula* and *Candona*. Although strongly resembling each other in valve morphology, there are some differences in size and valve outline between the specimens of *P. hainburgensis* described by Danielopol et al. (1991) and Gross and Piller (2006) (Figs 7, 8). The observed morphological variations can be ascribed either to intraspecific variability and/or occurrence of different species.

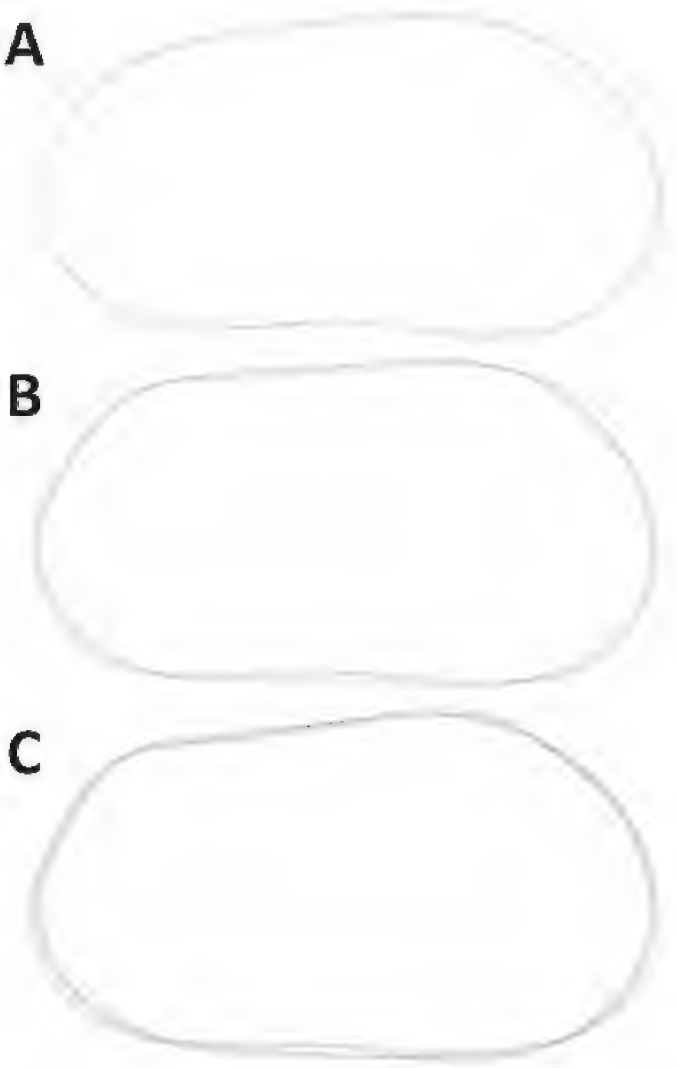

Figure 8. Superimposition of valve outlines of Recent and fossil species of *Pseudolimnocythere* in normalized area mode **A** "curved" valves: *P. hainburgensis* (from Danielopol et al. 1991 and Gross and Piller 2006) **B** "slightly inclined" valves: *P. sofiae* sp. nov., *P. hypogaea* (from Klie 1938), *P. hartmanni* (from Danielopol 1979) **C** "sloping" valves: *P. abdita* sp. nov., *P.* sp. (from Peterson et al. 2013), *P.* sp. (from Montanari et al. 2021), *P.* sp. sensu Schornikov, 2013 (from Schornikov et al. 2014); *P.* sp. (from Danielopol 1980).

Pseudolimnocyhtere sp. Nachite et al. 2003 (not figured)

The authors report seven specimens form the late Pliocene Saïss basin, Douar section, Northern Marocco. Possibly the same as *Pseudolimnocythere* sp. A Bekkali & Nachite, 2003 (see below).

Pseudolimnocythere sp. A (plate III–8 in Bekkali and Nachite 2006)

Late Pleistocene, Saïss plain, Douar section, in an old quarry of Douar El Mechmach on the road from Fès to Aïn Chkef Morocco. One left valve, length ~230 µm, either small-sized species or A-1 stage.

Pseudolimnocythere hartmanni Danielopol 1979

Cores drilled in the harbour of Salerno, Tyrrhenian Sea, Italy. Considered as an allochthonous species. It was found in sample GS1 7.50, Holocene fine sands and rare

Figure 9. Paleogeographic map of the Mediterranean basin and Western Paratethys during Midde Miocene (13 Ma, after Ron Blakey, Colorado Plateau Geosystems, Arizona, USA, http://cpgeosystems.com) and distribution of Recent (circles) and fossil (stars) species of *Pseudolimnocythere* **A** *P.* sp. (Danielopol and Bonaduce 1990) **B** *P. abdita* sp. nov. **C** *P. sofiae* sp. nov. **D** *P.* sp. (Peterson et al. 2013) **E** *P. hypogaea* (Karanovic and Pesce 2001) **F** and **G** *P. hypogaea* (Klie 1938) **H** *P.* sp. (Danielopol 1980) **I** *P.* sp. (Montanari et al. 2021) **J** *P. hartmanni* (Danielopol 1979) **K** *P.* sp. *sensu* Schornikov 2013 (Schornikov et al. 2014) **L** *P. hainburgensis* (Danielopol et al. 1991 **M** *P.* sp. (Bekkali and Nachite 2006) **N** *P. hartmanni* (sensu Aiello et al. 2020).

gravels of the unit US5 (in some levels of the unit US5 ceramic fragments of Roman age occur, and in the lower part is present a level dated at 7553–7411cal BP), and in sample GS1 16.50, Pleistocene, older than the Campanian Ignimbrite super-eruption (~40 ka), possibly the substage MIS 5e (Aiello et al. 2020).

The left valves of *P. hartmanni* illustrated in the literature (fig. 3 in Danielopol 1979 and fig. 6.9 in Aiello et al. 2020) have very similar length (0.29–0.30 mm), but the length/height ratios are 1.96 and 1.75, respectively. So far, it is impossible to establish whether these differences fall within the range of variability of *P. hartmanni*, or rather the specimens belong to different species. Due to the deteriorated margins of the valve, the specimen of *P. hartmanni* from Aiello et al. (2020) was not used for the analysis of the valve outlines. The record from Salerno, if confirmed, greatly expands the geographic range of *P. hartmanni*, previously known only for Euboea Island, Greece, as a living species.

Discussion

Pseudolimnocythere shows a pre-adaptation to groundwater lifestyle

Speciation in subterranean habitats is commonly explained as the result of divergent selection in geographically isolated populations (Konec et al. 2015; Mammola et al. 2018). The role of evolutionary conservatism and convergence arising from the selective pressure of groundwater environments result in similarity in stygobitic species traits over large spatial scales (Bauzà-Ribot et al. 2011). Small size, trapezoidal, triangular, or streamlined carapace shape and lack of swimming bristles are highly conservative morphological traits in stygobitic ostracods (Marmonier et al. 1994, Dole-Olivier et al. 2000).

Differences between extant *Pseudolimnocythere* species are mostly apparent in the structure of the male copulatory organ, while differences in other soft parts and valve morphology are limited to minor details. All known species of the genus, both fossil and extant, show substantial uniformity in size, with lengths in the 0.25–0.36 mm range. This morphological trait represents a pre-adaptation in carapace shape and size suitable for life in non-marine interstitial and porous or fissured groundwater aquifers. Carapace features show a remarkable evolutionary conservatism over time. The presence of different kinds of hinges is an exception to this valve morphological uniformity. It has been suggested that a complication of the hinge structure in loxoconchids, compared to the amphidont basic type, may be linked to an increase in the level of calcification of the carapace (Yamaguchi 2003).

Colonization and dispersal in continental groundwaters

The evolutionary origins of subterranean Recent ostracods are best understood as due to an interplay of vicariance and dispersal. Danielopol (1980) discussed in detail two main concurrent hypotheses to infer the antiquity of the genus *Pseudolimnocythere*. He applied the regression evolution model (Stock 1977) together with possible pathways of colonization of continental areas. Both hypotheses assumed that *Pseudolimnocythere* species colonized inland subterranean waters during marine regressions, ranging from the Upper Miocene – Lower Pliocene to Pleistocene. Afterward, Danielopol and Bonaduce (1990) suggested the Messinian salinity crisis as a key-event favouring *Pseudolimnocythere* colonization. Although at that time the known *Pseudolimnocythere* species had been found exclusively near the present coastline of the Mediterranean Sea, Danielopol (1980) hypothesized the possible discovery of *Pseudolimnocythere* species far away from coastlines due to their presumed ability of dispersal, mainly due to their small size. However, the subsequent discovery of Miocene (Danielopol et al. 1991) and Quaternary fossil and sub-fossil species (Bekkali and Nachite 2006; Aiello et al. 2020), and of further Recent species in continental groundwaters (Peterson et al. 2013; this paper) have brought useful elements to better hypothesize the time and mode of colonization routes of *Pseudolimnocythere*.

As reported by Savatenalinton and Martens (2009), the hypothesis by Danielopol and Bonaduce (1990) of a main colonization event during the Messinian salinity crisis must be rejected, because there were already non-marine *Pseudolimnocythere* species in the Middle Miocene. Furthermore, Danielopol et al. (1991) contradicted the Messinian salinity crisis hypothesis for *P. hainburgensis*, a freshwater or brackish water species from the Badenian of the Vienna Basin, pointing out that this colonization must have taken place before. The Vienna Basin was subject to an important sea-level drop associated with an Antarctic cooling step 14.2 Ma (Rögl et al. 2007). More generally, the Middle Miocene of Europe was a time of paleogeographic reorganizations and strong tectonic activities, due to the orogeny and uplift of the Alpine-Himalayan chains and global climate change (Zachos et al. 2001). These changes also affected coastal marine environments, together with the intermittent opening and closing of marine waterways between Indian Ocean, Mediterranean, and Paratethys (Rögl 1999; Sant et al. 2017).

Moreover, *Pseudolimnocythere* sp. A Bekkali and Nachite 2003 from the Plio-Pleistocene basin of Saïss was associated with a true freshwater fauna. The presence of taxa such as *Candona angulata*, *Fabaeformiscandona fabaeformis*, *Potamocypris* sp. and *Darwinula* sp. indicated freshwaters rather rich in bicarbonate and slightly alkaline; these conditions are probably due to water coming from the Lias limestone and dolomite beds bordering the basin (Bekkali and Nachite 2006).

Recently, different biogeographical models have been explored to explain the distribution in the groundwaters of the Apennine and Balkanic peninsula, separated each other by the Adriatic Sea, of three different clades of stygobitic amphipods of the genus *Niphargus* Schiödte 1849. Among the considered scenarios, marine regression/transgression cycles resulted to be the most relevant events explaining their trans-Adriatic distribution, while transitional freshwater subterranean pathways created by landmass connections probably did not play an important role (Delić et al. 2020). The known distribution of the fossil and living species of *Pseudolimnocythere* seems to confirm this kind of vicariance model for this thalassoid genus, and the occurrence of repeated and independent events of colonization of continental waters linked to sea level variations since the Middle Miocene, can be hypothesized. Distributional data compared to Middle Miocene coastlines (Fig. 9) show a quite good correspondence between present distribution and the ancient coastlines, suggesting a previous dispersal stage along shorelines followed by multiple invasion routes rather than a single colonization event followed by dispersal. A further migration from subsurface interstitial to deeper groundwater habitats is of course possible.

The occurrence of repeated and independent colonizations and local speciation events seems to be further supported by the finding of *Pseudolimnocythere* species only in their type localities or, for *P. hypogaea*, in a small area, indicating that their dispersal ability is quite low. The only notable exception could be *P. hartmanni*, found in the Quaternary of southern Italy, and as extant species in a Greek Aegean Island, albeit this conspecificity can be questioned and needs to be confirmed.

Furthermore, it is worth noting that most *Pseudolimnocythere* species occur in correspondence with carbonate outcrops (Suppl. material 1: Fig. S5). Submarine karstic

springs in shallow marine areas have been proposed as convenient pathways for the colonization of inland subterranean habitats by marine lineages because their lower concentration of Na^{+} ions typical of sea water is compensated by a higher concentration of Ca^{2+} ions (Danielopol and Bonaduce 1990; Notenboom 1991). This fact suggests that the karstic micro-crevices may be one of the main habitats exploited by *Pseudolimnocythere* species, as was clearly demonstrated in the study of Poiano karstic springs for *P. sofiae* sp. nov. (Stoch et al. 2009a).

Osmoregulatory mechanisms that make it possible to cross the salinity boundary (Aladin and Potts 1996) are of fundamental importance for the colonization of inland groundwater by marine ancestors. There are euryhaline Cytheroidea species living on the surface of shallow marine sediments which can be found in brackish and freshwater habitats too, like *Cyprideis torosa* Jones, 1950 (Meisch 2000). The ability to move upstream in small lotic environments is documented for some cytherid species, as *Tyrrhenocythere amnicola* (Sars, 1887) as shown by Pieri et al. (2015, 2020). Among living representatives of *Pseudolimnocythere*, possibly only *P. abdita* sp. nov. lives in freshwater, while the other extant species of this genus were found in habitats influenced by marine waters, with a salinity gradient form brackish to saline, or in sodic (as *P. sofiae* sp. nov.) and sulfidic (as *P.* sp. Peterson, 2013) waters. As reported above, also the fossil species *P. hainburgensis* and *P.* sp. A Bekkali & Nachite, 2003 were able to invade low-salinity environments.

Negative rheotaxis has been observed in different meiofaunal taxa, demonstrating movements directed upstream in the hyporheic zone (Bruno et al. 2012). For example, the presence of *P. abdita* sp. nov. at over 1000 m above sea level can be explained by the relative proximity of the crest of northern Apennine chain to the coastline during the late Pliocene, and by the presence of small watercourses which may have served as routes of colonization of groundwater ecosystems. Transport of stygobionts from inland subterranean habitats through surface or sub-surface outflow is another possible dispersal mechanism. A survey carried out at Poiano spring demonstrated that rapid discharge variation due to rainfall was responsible for the drift of up to 38 individuals per day of *Pseudolimnocythere sofiae* sp. nov. (Stoch et al. 2009a). However, the scattered records of individuals (mostly larvae) found downstream in the Secchia river, did not evidence the actual colonization of the interstitial habitats, indicating that the role of this mechanism may be of minor importance, even at a very small spatial scale.

Conclusions

Our analyses have been directed toward reassessing existing theories on evolution and biogeographic patterns of subterranean non-marine ostracods, based on the known distribution and new findings of species belonging to the genus *Pseudolimnocythere*. This allowed us to hypothesize different evolutive paths determined by geological events as well as by species morphological and ecological traits and their responses to selective pressures.

Limited accessibility to hypogean habitats determines a still fragmented knowledge of groundwater ostracod biodiversity. In addition, poor taxonomic resolution often hinders the possibility to identify patterns and scenarios at suitable space and time scales. Extensive research in hypogean environments will significantly increase our poor knowledge on ostracod diversity in groundwater and will contribute to a better understanding of their evolutionary and systematic relationships. Up to now, the rarity and difficulty to collect *Pseudolimnocythere* species prevented the application of DNA sequencing in studying this peculiar genus. There is no doubt that, along with a traditional morphological approach (indispensable when comparing fossil and Recent species), the use of molecular markers will disclose new possibilities for the investigation of the evolutionary history of subterranean ostracods.

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Supplementary material 1

Figures S1–S5

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Data type: Occurrences and images

Explanation note: Additional information on the sampled localities and the geographycal distribution of the genus *Pseudolimnocythere*.

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